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International application number: PCT/GB05/000225

International filing date: 21 January 2005 (21.01.2005)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/560,336
Filing date: 06 April 2004 (06.04.2004)

Date of receipt at the International Bureau: 15 February 2005 (15.02.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

World Intellectual Property Organization (WIPO) - Geneva, Switzerland
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APPLICATION NUMBER: *60/560,336*
FILING DATE: *April 06, 2004*

GB/05/225

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. EL972392164US

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Preben	Lexow	Oslo, Norway

Additional inventors are being named on the _____ separately numbered sheets attached hereto

TITLE OF THE INVENTION (500 characters max)

Method of Analysis

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

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OR

☐ Firm or Individual Name					
Address					
Address					
City		State		ZIP	
Country		Telephone		Fax	

ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification *Number of Pages* 5

☒ Drawing(s) *Number of Sheets* 1

☐ Application Data Sheet. See 37 CFR 1.76

☐ CD(s), Number _____

☐ Other (specify) _____

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☒ Applicant claims small entity status. See 37 CFR 1.27.

☐ A check or money order is enclosed to cover the filing fees.

☒ The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: 19-0065

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FILING FEE AMOUNT ($): $80.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are: _____

Respectfully submitted,

SIGNATURE Glenn P. Ladwig

TYPED or PRINTED NAME Glenn P. Ladwig

TELEPHONE (352) 375-8100

Date April 6, 2004

REGISTRATION NO. 46,853 *(if appropriate)*

Docket Number: GJE-1013P

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

This collection of information is required by 37 CFR 1.51. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Mail Stop Provisional Application, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

Provisional Patent Application
Docket No. GJE-1013P

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Docket No. : GJE-1013P

Applicant : Preben Lexow

For : Method of Analysis

MS PROVISIONAL PATENT APPLICATION
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313

CERTIFICATE OF MAILING BY EXPRESS MAIL (37 C.F.R. § 1.10)

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I hereby certify that the items listed on the attached Provisional Application and Cover Sheet therefore, with copies as required for authorization for use of Deposit Account No. 19-0065, are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. § 1.10 on the date indicated above and are addressed to: Mail Stop PROVISIONAL PATENT APPLICATION, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313.

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Method of Analysis

Field of Invention

The present invention relates to methods for improving polynucleotide ligation reactions.

Background to Invention

Berg and Boyer created the first recombinant DNA molecule in 1972. This simple concept of recombination - the splicing together of two pieces of DNA and fusing them by ligation, is the basis for the entire field of molecular biology. Molecular biology has become ubiquitous to the point where it is central to the majority of all biological research. The ligation reaction is performed thousands of times a day in research and diagnostic laboratories worldwide. Given the boundless opportunity presented by genetic engineering, the ligation reaction is likely to remain a central technique for many years to come.

The ligation reaction itself is chemically simple, comprising the linking of two nucleotides by the creation of a phosodiester bond between the 3' hydroxyl of one nucleotide and the 5' phosphate of another, by a ligase enzyme. There are two types of ligation, known as "sticky end" and "blunt end", depending on the presence or lack (respectively) of complementary single stranded regions on the two polynucleotides to be joined, in proximity to the ligation location. "Sticky-end" ligations involve the hybridisation of complementary single stranded sequences between the two polynucleotides to be joined, prior to the ligation event itself. Sequences that have similar but not 100% complementary single stranded sequences will still be ligated, known as a mismatch ligation. These result in the ligation of an incorrect sequence and decrease the efficiency and fidelity of the overall ligation reaction.

Since ligation is such an important reaction, ligases are available on the market that are improved, modified and optimised to give maximum efficiency. These enzymes are expensive and it is therefore desirable to use as a small amount as is possible without reducing the efficiency of the reaction and whilst avoiding mismatch ligation. Mismatch ligations are problematic as they are deleterious to the fidelity of the ligation process.. It is therefore desirable to minimise mismatch ligations.

Current methods of increasing ligation specificity include decreasing the amount of ligase and increasing the salt in the reaction mix to slow down the reaction. Since match ligations are much faster than mismatch ligations, the increased specificity observed using this technique is a result of the slower reaction speed and whilst this increases the match: mismatch ratio, it results in a low yield and does not prevent mismatch ligations.

Summary of Invention

The present invention is based on the realisation that specificity in "sticky-end" ligations can be increased by including short adapters that reduce the occurrence of mismatch ligation.

According to a first aspect of the invention, a method for improving the binding between a first double stranded polynucleotide having a single stranded portion and a second polynucleotide having a complementary single stranded portion, said second polynucleotide being present in a sample comprising a mixture of different polynucleotides, comprises:

contacting the sample, under hybridising conditions, with the first polynucleotide and one or more third polynucleotide(s), wherein the third polynucleotide(s) comprises a single stranded portion that differs from the single stranded portion of the first polynucleotide by at least one base substitution:

The present invention improves the yield of match ligations by reducing mismatch ligations through the use of blocking polynucleotides which hybridise incorrect single stranded overhangs.

Description of the Drawings

The present invention is illustrated by reference to the accompanying drawing, where:

Figure 1 is a graphic illustration of match:mismatch ratio as a function of time.

Detailed Description of the Invention

The present invention is used to increase specificity of polynucleotide ligations. The term "polynucleotide" is used herein to refer to biological molecules made up of a plurality of nucleotides. Preferred polynucleotides include DNA, RNA and synthetic analogues thereof, including PNA.

The term "hybridising conditions" is used herein to refer to conditions that allow complementary base pairing to occur between two polynucleotides, such that two complementary single stranded polynucleotides will hybridise to form a duplex. Such conditions are well known in the art. An Example of such conditions is incubation at 42°C in a solution comprising: 50% formamide, 5x SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 µg/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65°C.

In any ligation reaction, two polynucleotide molecules are joined. The term "first polynucleotide" is used herein to refer to one of the two intended targets of ligation. The term "second polynucleotide" is used herein to refer to the other of the two intended targets of ligation.

A non-limiting example of the terms "first polynucleotide" and "second polynucleotide" comprises the "first polynucleotide" being a DNA vector into which an insert, the "second polynucleotide", is to be ligated to form a recombinant construct.

In any ligation reaction, there may be polynucleotides present which are neither "first polynucleotides" or "second polynucleotides". These polynucleotides interfere with the ligation between the first and second polynucleotides, which results in mismatch ligations. As used herein, the term "third polynucleotide" is used to describe polynucleotides which are added to the ligation reaction mixture to hybridise to any polynucleotide which is not a first or second polynucleotide, preventing the unwanted polynucleotides from reacting with the other components of the reaction mix. The third polynucleotides are not totally complementary to the first or second polynucleotides.

The method increases specificity in polynucleotide ligations through the addition of one or more third polynucleotide(s) into a reaction mix. This reaction mix comprises a first polynucleotide and a second polynucleotide, which contain complementary single stranded portions. The second polynucleotide is present in a sample comprising a mixture of different polynucleotides, of which only one ("the second polynucleotide") is the correct target for binding to the first

polynucleotide. The third polynucleotide(s) comprises a single stranded portion that differs from the single stranded portion of the first polynucleotide by at least one base. The number of differences between the first and third polynucleotides may depend on the size of the single stranded portions involved. For example, if the single stranded portion is only 3 bases in length, a single difference may be suitable, but if the single stranded portion is 6 bases in length, multiple differences may be preferred. The differences may be substitution(s), deletion(s) or addition(s).

The third polynucleotide may be added to the sample containing the second polynucleotide simultaneously with or sequentially before or after the first polynucleotide. The third polynucleotide is preferably added to the sample containing the second polynucleotide, along with the first polynucleotide.

Preferably, the third polynucleotide is present in excess with respect to the first and second polynucleotides, to ensure that all other polynucleotides in the sample are hybridised by the third polynucleotide.

It is intended that the first and second polynucleotides hybridise and are ligated together, to the exclusion of other polynucleotides in the sample. The third polynucleotide(s) hybridise to the other polynucleotides in the sample which would otherwise compete for binding to the first polynucleotides, effectively preventing them from hybridising to the first polynucleotides and increasing the number of correct binding events between the first and second polynucleotides. This increases the efficiency and yield of the overall ligation reaction.

Preferably the mixture of third polynucleotides comprises double stranded polynucleotides with a single stranded portion, such that the single stranded portion hybridises its complementary region on incorrect first and second target polymers.

Preferably, the single stranded portion of each of the first, second and third polynucleotides is from 3 to 6 bases in length. Most preferably, the single stranded portion is 4 bases in length.

Figure 1 is a graphical representation of the match:mismatch ratio as a function of time. This ratio becomes lower as the reaction progresses, since the match reaction rapidly reaches plateau and is caught up by the slower mismatch

reaction. Traditional methods of increasing specificity merely slow the reaction (using less ligase or increasing salt concentration) and shift the reaction to the left of the graph, where the match:mismatch ratio is favourable but yield is decreased. The present invention ensures that the mismatch ligations do not increase. The match reaction can proceed to full term without the mismatch reaction ever catching up. This gives improved yield and optimised match:mismatch ratio.

In one aspect, the present invention provides a method for improving the binding between a first double stranded polynucleotide having a single stranded portion and a second polynucleotide having a complementary single stranded portion, wherein the second polynucleotide is present in a sample comprising a mixture of different polynucleotides, the method comprising contacting the sample, under hybridizing conditions, with the first polynucleotide and one or more third polynucleotide(s), wherein the third polynucleotide(s) comprises a single stranded portion that differs from the single stranded portion of the first polynucleotide by at least one base substitution. The third polynucleotide can be a double stranded polynucleotide having the single stranded portion.

Preferably, the single stranded portion on each of the first, second, and third polynucleotides is from 3 to 6 bases in length. More preferably, the single stranded portion is 4 bases in length.

The single stranded portion of the third polynucleotide can differ from the single stranded portion of the first polynucleotide by one or more bases. In a specific embodiment, the single stranded portion of the third polynucleotide differs from the single stranded portion of the first polynucleotide by one base.

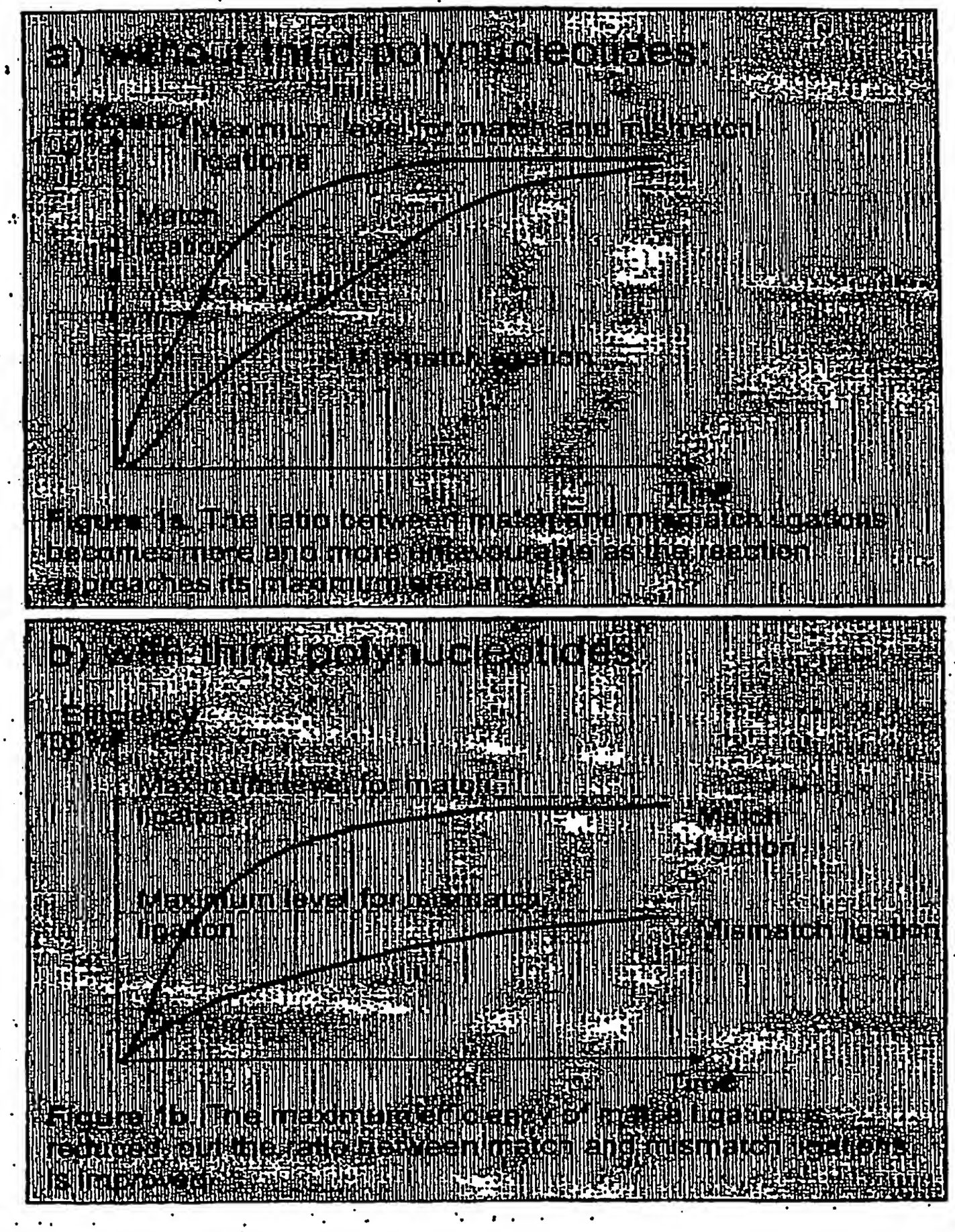

Figure 1

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(PCT Administrative Instructions, Section 411)

To:

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Broadgate House
7 Eldon Street
London EC2M 7 LH
ROYAUME-UNI

Date of mailing *(day/month/year)* 23 March 2005 (23.03.2005)	
Applicant's or agent's file reference JWJ01013WO	**IMPORTANT NOTIFICATION**
International application No. PCT/GB05/000225	International filing date *(day/month/year)* 21 January 2005 (21.01.2005)
International publication date *(day/month/year)*	Priority date *(day/month/year)* 23 January 2004 (23.01.2004)
Applicant LINGVITAE AS et al	

1. By means of this Form, which replaces any previously issued notification concerning submission or transmittal of priority documents, the applicant is hereby notified of the date of receipt by the International Bureau of the priority document(s) relating to all earlier application(s) whose priority is claimed. Unless otherwise indicated by the letters "NR", in the right-hand column or by an asterisk appearing next to a date of receipt, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).

2. *(If applicable)* The **letters "NR"** appearing in the right-hand column denote **a priority document which, on the date of mailing of this Form, had not yet been received by the International Bureau** under Rule 17.1(a) or (b). Where, under Rule 17.1(a), the priority document must be submitted by the applicant to the receiving Office or the International Bureau, but the applicant fails to submit the priority document within the applicable time limit under that Rule, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

3. *(If applicable)* **An asterisk (*)** appearing next to a date of receipt, in the right-hand column, denotes **a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b)** (the priority document was received after the time limit prescribed in Rule 17.1(a) or the request to prepare and transmit the priority document was submitted to the receiving Office after the applicable time limit under Rule 17.1(b)). Even though the priority document was not furnished in compliance with Rule 17.1(a) or (b), the International Bureau will nevertheless transmit a copy of the document to the designated Offices, for their consideration. In case such a copy is not accepted by the designated Office as the priority document, Rule 17.1(c) provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

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23 January 2004 (23.01.2004)	0401525.1	GB	25 February 2005 (25.02.2005)
06 April 2004 (06.04.2004)	60/560,336	US	15 February 2005 (15.02.2005)

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